Suppression of Verticillium Wilt of Tomato by Difluoromethylornithine, a Suicidal Inhibitor of Polyamine Biosynthesis

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ABSTRACT

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Difluoromethylornithine (DFMO), a specific inhibitor of polyamine biosynthesis via ornithine decarboxylase, retarded mycelial growth of *Verticillium dahliae* in vitro at concentrations as low as 5 μM. This inhibition could be reversed by putrescine, indicating that the mechanism of inhibition was prevention of polyamine biosynthesis in the mycelium. Postinoculation foliar application of DFMO delayed the appearance of symptoms of Verticillium wilt, and increasing doses of the inhibitor reduced the severity of the disease. These results indicate that it may be possible to control Verticillium wilts with target-specific inhibitors such as DFMO.

Polyamines have been demonstrated to be essential for optimal growth of animals, plants, and fungi (11,12). The synthesis of polyamines has been shown to proceed through two pathways, one involving ornithine decarboxylase (ODC) and the other utilizing arginine decarboxylase (ADC) (11). Although both the ODC and ADC pathways have been demonstrated in plant and bacterial cells, fungi apparently possess only the ODC pathway (7). Because the primary pathway for polyamine biosynthesis in plants is via ADC (11), and most fungi apparently do not possess this pathway, it is possible that plant diseases of fungal origin might be controlled by specific inhibition of the ODC pathway (7). The availability of α-difluoromethylornithine (DFMO) and α-difluoromethylarginine (DFMA), specific, enzyme-activated, irreversible (suicidal [2]) inhibitors of ODC and ADC, respectively (2,3,5), has made it possible to test this possibility.

Rajam and Galston (7) investigated the effects of DFMO and DFMA on mycelial growth of several fungal plant pathogens. They found that these materials inhibited vegetative growth of the fungi and that this inhibition could be reversed by adding polyamines such as putrescine or spermidine to the culture media. Rajam et al (8) investigated the

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effects of DFMO and DFMA on bean rust, caused by *Uromyces phaseoli*, and found that DFMO, but not DFMA, would prevent the occurrence of this disease.

We report the results of studies on the effects of DFMO on mycelial growth of *Verticillium dahliae* in vitro and on symptom expression in Verticillium wilt of tomato.

MATERIALS AND METHODS

A tomato isolate of *V. dahliae*, TS-1B, was maintained on Czapek-Dox nutrient agar by biweekly transfers from the leading edge of the culture. Spores for inoculation of tomato plants were produced by modified hanging drop techniques (6), incubating the spore cultures at 20 C under a 16-hr photoperiod provided by 60 μE s^{-1} m^{-2} of mixed fluorescent and incandescent light. In vitro studies involved Czapek-Dox nutrient salts containing 1% glucose, solidified with 0.75% agarose (IBI Inc., New Haven, CT), and supplemented with inhibitors and/or putrescine added to the molten medium by filtration through 0.45-μ filters. Mycelial growth was determined in 6-cm petri dishes containing 7.5 ml of the appropriate culture medium. Radial growth was determined after 8 days of incubation in darkness at 20 C by averaging measurements made at right angles across the diameters of the developing colonies.

Tomato seedlings (*Lycopersicon esculentum* Mill. 'Bonny Best') were cultured in 4-in. pots of Boyce Thompson potting soil under standard greenhouse conditions involving a 16-hr photoperiod provided by a mixed bank of fluorescent and incandescent lights, with temperatures of 23 C during the day and 20 C at night. At the five-leaf stage, plants were inoculated by applying 5 ml of 10^4 spores per milliliter per pot (25 propagules per gram of soil). At the times indicated, plants were sprayed to runoff with either DFMO or DFMA formulated in 0.01% Tween 20, pH 7.0. Control plants were sprayed similarly with Tween 20. Disease expression was evaluated using a subjective scale of 0–5, where 0 = no disease symptoms and 5 = dead plant.

ODC and ADC activity determinations were carried out on 12-day-old mycelium of the fungus cultured on liquid Czapek-Dox medium containing 1% glucose as the carbon source. ODC and ADC were assayed by the methods of Kaur-Sawhney et al (4), using D,L-[1-^{14}C]-ornithine and L-[U^{14}C]arginine as substrates for the respective enzymes. Arginase activity was determined by the methods of Schimke (9), using arginine and DFMA as substrates and 1-phenyl-1,2-propanedione-2-oxime to detect the urea released by the enzyme. Controls for the arginase determinations were boiled mycelial extracts, and results were adjusted to compensate for readings obtained from substrate blanks. Protein determinations were by the Bradford method (1), using bovine serum albumin as a reference standard. DFMO and DFMA were gifts from Merrell-Dow; all other reagents were from Sigma Biochemicals. All experiments were replicated three times and repeated twice. Variations within experiments were generally less than 5%.

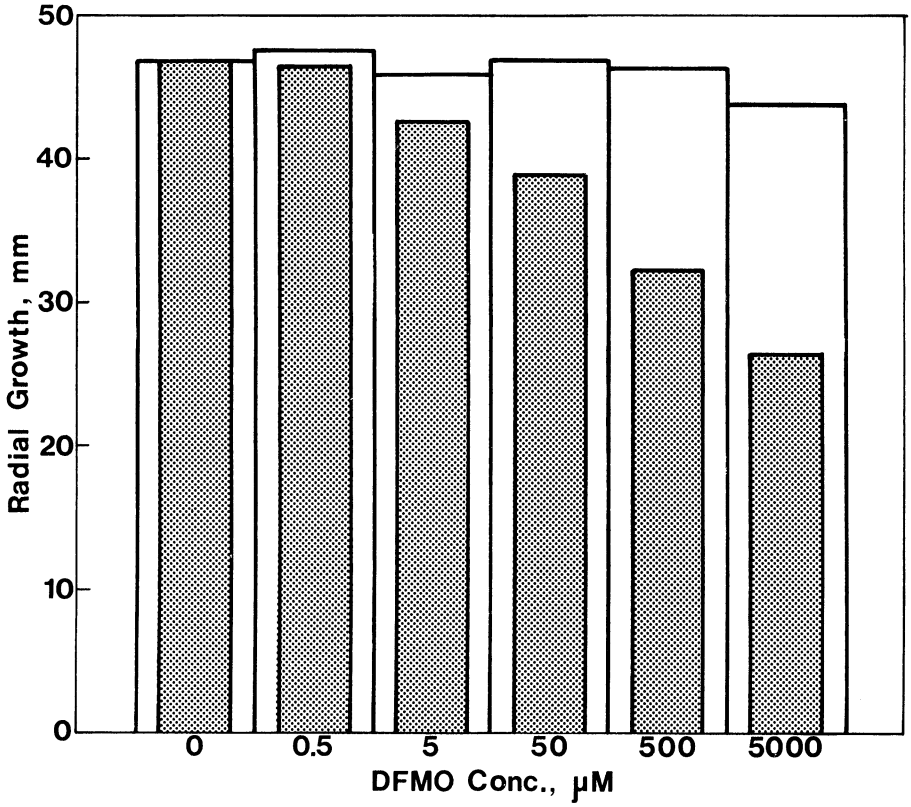

Fig. 1. Inhibition of radial growth of *Verticillium dahliae* by increasing concentrations of α-difluoromethylornithine (DFMO) (shaded bars) and reversal of this inhibition by 1 mM putrescine (open bars). Data from 8-day-old cultures.

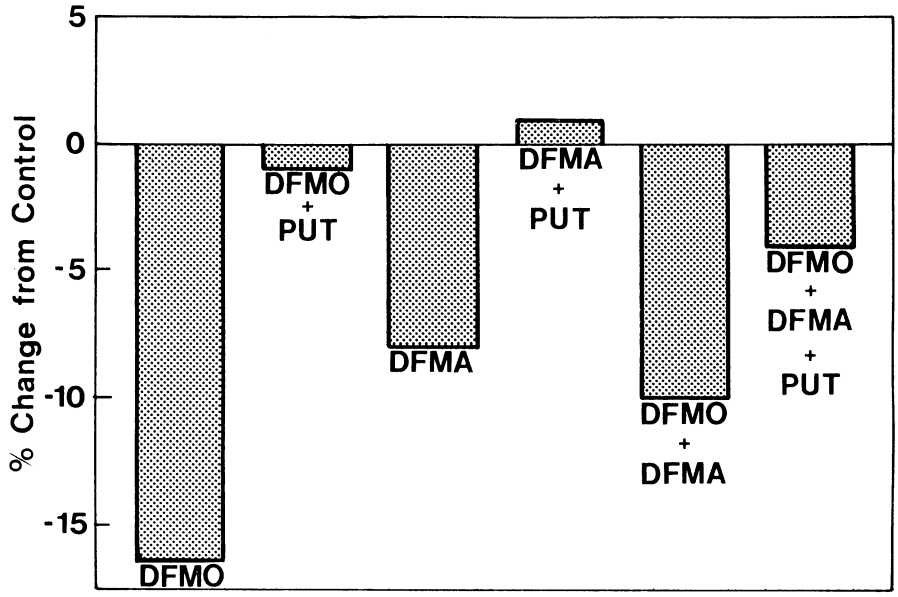

Fig. 2. Inhibition of radial growth of *Verticillium dahliae* induced by 500 μM α-difluoromethylornithine (DFMO) and α-difluoromethylarginine (DFMA), alone and combined, and reversal of this inhibition by 1 mM putrescine (PUT). Data from 8-day-old cultures.

RESULTS AND DISCUSSION

Radial growth of *V. dahliae* in vitro was reduced by concentrations of DFMO above 5 μM (Fig. 1), and this inhibition could be reversed by adding 1

mM putrescine to the culture medium, indicating that the mechanism of DFMO-induced growth inhibition was probably through inhibition of polyamine biosynthesis via ODC. In addition, higher concentrations of DFMO (500 μM and higher) inhibited pigment synthesis by this fungus, and this inhibition of pigment synthesis could also be reversed by adding putrescine to the medium. When the inhibitory effects of DFMO and DFMA were compared in vitro, DFMA appeared to inhibit radial growth of the fungus to about 50% of that observed with DFMO (Fig. 2). These results are similar to those obtained by Rajam and Galston (7) with several other phytopathogenic fungi. These authors suggested that this DFMA-induced inhibition might be due to enzymatic conversion of DFMA to DFMO by arginase. We have examined the levels of ODC, ADC, and arginase in extracts from the vegetative mycelium of *V. dahliae*, and our results (Table 1) indicate that this may indeed be the case. Both isolates of *V. dahliae* examined contained substantial levels of ODC activity and little or no ADC activity. The apparent ADC activity, if indeed it was ADC activity, was extremely low, and this small amount of apparent activity could have been due to other aspects of arginine metabolism (10). When assayed with arginine, extracts from the vegetative mycelium of tomato isolates of *V. dahliae* contained detectable arginase activity (Table 1). The enzyme was also capable of generating urea from DFMA (Table 1), and in the process, presumably converting DFMA to DFMO as suggested by Slocum and Galston (10). Although the levels of arginase detected in the mycelium were low, this activity is apparently adequate to convert sufficient DFMA to DFMO for partial inhibition of in vitro radial growth of the fungus. The fact that the inhibitory effects of DFMA were not additive when both DFMO and DFMA were incorporated into the culture medium also suggests that the in vitro effects of these two materials are due to inhibition at the same metabolic site.

Our first in vivo investigation involved an attempt to determine the optimal timing of treatment with respect to inoculation to obtain maximum suppression of the symptoms of Verticillium wilt. This experiment indicated that, as has been observed with bean rust (8), the most effective times for application of the inhibitor were 24–48 hr postinoculation (Fig. 3). Using this information, we examined disease progress in plants treated with two levels of DFMO 24 hr after inoculation with *V. dahliae*. The results of this experiment (Fig. 4) indicated that DFMO, at both 2.5 and 10 mM, suppressed symptoms of Verticillium wilt of tomato compared with the controls. Our results also indicated that

Table 1. Activities of ODC, ADC, and arginase observed in extracts from mycelium of 12-day-old cultures of *Verticillium dahliae*[a]

	Enzyme activity			
			Arginase[c]	
Isolate	ODC[b]	ADC[b]	ARG	DFMA
TS-1B	395.9	1.2	11.2	7.1
TS-2B	190.6	8.0	19.1	11.3

[a] ODC = ornithine decarboxylase, ADC = arginine decarboxylase, DFMA = α-difluoromethylarginine, and ARG = arginine.
[b] pM CO_2/hr/mg protein.
[c] pM urea/hr/mg protein.

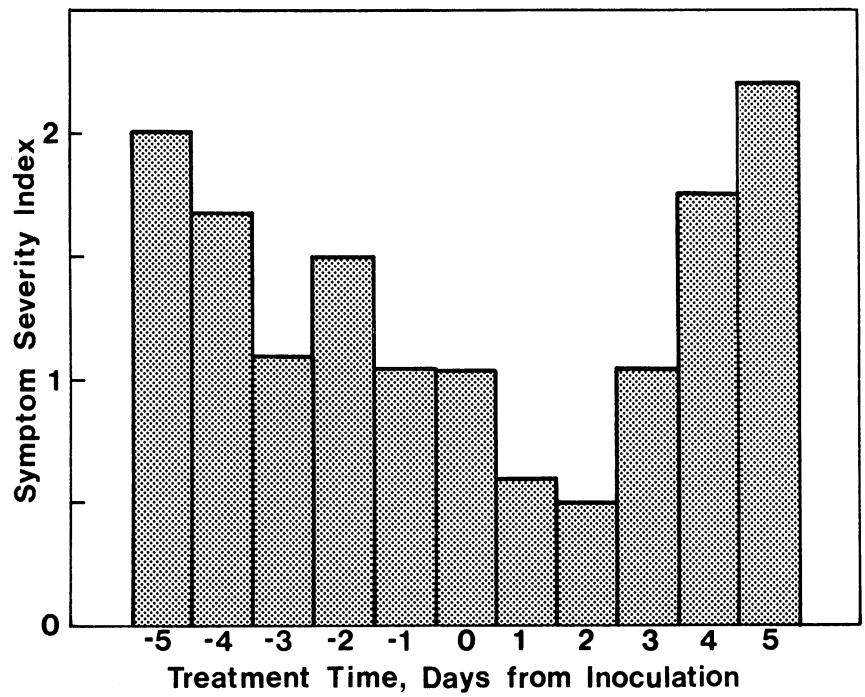

Fig. 3. Influence of treatment time on the effects of 5 mM α-difluoromethylornithine (DFMO) on Verticillium wilt symptoms in Bonny Best tomato 21 days after inoculation.

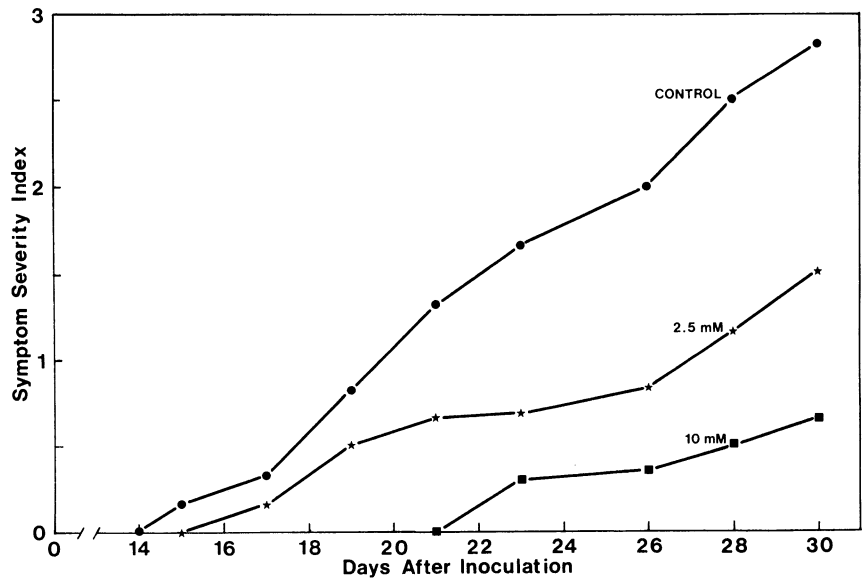

Fig. 4. Inhibition of the disease progress curve for Verticillium wilt of Bonny Best tomato by 2.5 and 10 mM foliar application of α-difluoromethylornithine (DFMO).

increasing the concentration of DFMO from 2.5 to 10 mM delayed the onset of symptom expression up to 7 days. In a similar series of experiments, we determined that DFMA, at concentrations up to 10 mM, did not alter either symptom severity or time of symptom expression in this disease. Although not investigated, the ineffectiveness of DFMA in vivo can probably be attributed to binding of the material to tomato ADC, rendering the DFMA unavailable to the arginase of the pathogen. When applied to uninoculated tomato plants at concentrations up to 20 mM, DFMO had no apparent effects on the growth and development of the plants for the duration of the 28-day observation period.

The results of these and other studies (7,8) indicate that the in vitro sensitivity of phytopathogenic fungi to DFMO varies widely and may not be a reliable indicator of the potential efficacy of the materials for disease control. The results of our in vivo experiments indicate that target-specific inhibitors of polyamine biosynthesis, like DFMO, may prove useful for control of fungal vascular diseases such as Verticillium wilt. These results also clearly indicate that inhibitors such as DFMO can effectively suppress disease symptoms even when applied to a part of the host plant that is spatially separated from the site of infection. Further work with these and other suicidal inhibitors of polyamine biosynthesis may result in the generation of a new group of target-specific pesticides for use in plant disease control.

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